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Identification and field evaluation of (E)-11,13-tetradecadienal as sex pheromone of the strawberry tortrix (*Acleris comariana*)

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Abstract

The strawberry tortrix (*Acleris comariana* Lienig and Zeller) (Lepidoptera: Tortricidae), is a major pest of strawberry in Denmark and southern Sweden. Chemical and electrophysiological analyses revealed a single compound, (E)-11,13-tetradecadienal (E11,13-14:Ald), in gland extracts of females eliciting a strong antennal response in conspecific males. Also (Z)-11,13-tetradecadienal (Z11,13-14:Ald) was found to be antennally active, but not detected in gland extracts. The corresponding alcohol and acetate of E11,13-14:Ald, which are biologically active in other *Acleris* species, were not produced by females and did not trigger electrophysiological response in males. Trapping experiments at a commercial strawberry farm in southern Sweden showed that E11,13-14:Ald and Z11,13-14:Ald, alone or in combination, attracted large numbers of males. Trap catches increased with increasing dose of E11,13-14:Ald, with traps baited with 100 µg and 1,000 µg being most attractive. Our results confirm the widespread use of E11,13-14:Ald as a key sex pheromone component in the genus *Acleris*. The identification of a highly attractive sex pheromone is a first step in developing pheromone-based methods for monitoring and control of *A. comariana* in European strawberry production.

KEYWORDS

electrophysiology, field trapping, gland extract analysis, pest management, strawberry pest

1 | INTRODUCTION

Acleris (Hübner) is a relatively large genus in the moth family Tortricidae with approximately 250 described species, including several important pests. A female-produced sex pheromone has been identified in four species: the yellowheaded fireworm (*A. minuta* Robinson; Schwarz, Klun, Hart, Leonhardt, & Weatherby, 1983), the western blackheaded budworm (*A. gloverana* Walsingham; Gray, Shepherd, Gries, & Gries, 1996), the eastern blackheaded budworm (*A. variana* Fernald) (Gries et al., 1994), and the yellow tortrix (*A. fimbriana* Thunberg) (Liu & Meng, 2002). Females of all four species produce the same gland

constituent, (E)-11,13-tetradecadienal (E11,13-14:Ald), to attract conspecific males. For both *A. gloverana* and *A. variana*, (E)-11,13-tetradecadienol (E11,13-14:OH) and (E)-11,13-tetradecadienyl acetate (E11,13-14:OAc) are also produced by females and elicit antennal response in males, but addition of these to E11,13-14:Ald does not enhance attraction in field trapping experiments (Gray et al., 1996; Gries et al., 1994). In contrast, addition of (Z)-11,13-tetradecadienal (Z11,13-14:Ald) to E11,13-14:Ald increases trap catches of *A. gloverana* (Gray et al., 1996). For *A. fimbriana*, E11,13-14:OAc increases attraction of males, whereas E11,13-14:OH has an inhibitory effect on males when combined with E11,13-14:Ald (Liu & Meng, 2002). Finally, four *Acleris* species are attracted to

E11,13-14:Ald, alone or in combination with (E)-11-tetradecenal, in Japan (Ando, Koike, Uchiyama, & Kuroko, 1987), showing the widespread use of E11,13-14:Ald as pheromone component in the genus.

The strawberry tortrix (*A. comariana* Lienig and Zeller) is widely distributed in Eurasia. This moth is a key pest in strawberry production in southern Sweden and Denmark (Lindhardt et al., 2003; Swedish Board of Agriculture, 2015). The species is the most commonly found tortricid on strawberries (Alford, 1984; Bovien & Thomsen, 1950; Solomon et al., 2001; Stenseth, 1982). In a recent survey conducted in organic and conventional Danish strawberry fields, about 95% of the collected tortricid larvae were identified as *A. comariana* (Sigsgaard et al., 2014). It has earlier been reported as a pest of strawberry in northwestern Germany (Heddergott, 1955) and England (Alford & Dockerty, 1974). This morphologically highly variable species is bivoltine, and in Sweden and Denmark the spring generation flies in June–July and the summer generation flies in August–October. Females lay their eggs underneath the leaves of the strawberry plant. Eggs overwinter and larvae of the spring generation often hatch at the time of flower petiole development. Feeding on flowers results in either aborted fruits or small, malformed fruits, which are generally rejected by consumers. Today, visual assessment is used to monitor larval densities of *A. comariana* in local fields, which is time-consuming and limits the possibility to reliably track population fluctuations over multiple years. Here, we report the identification of the sex pheromone of *A. comariana*, and development of a highly efficient trap lure, which can greatly facilitate detection and monitoring of this pest in strawberry production.

2 | MATERIALS AND METHODS

2.1 | Collection and rearing of moths

Strawberry leaves infested by *A. comariana* were collected from a 16 ha commercial field near Lockarp (55°31'N, 13°03'E), and a 2 ha commercial field near Flädie (55°44'N, 13°03'E), Skåne Province, Sweden, during May and August 2018. The leaves were arranged into bouquets tightly wrapped with a plastic bag, just slightly open in the top. The still attached leaf petioles were placed in water so that the leaves could remain fresh meanwhile the larvae continued their development. Leaves and larvae were transferred to transparent Plexiglas cages (30 cm × 30 cm × 60 cm), with a fine mesh net on the back side, and placed in a climate chamber at 20°C, 65% r.h., and a 17:7 L:D photoperiod. Pupae were separated by sex and kept in separate plastic boxes until adult emergence.

2.2 | Chemicals

Compounds previously identified from other *Acleris* species were purchased from Pherobank (Wijk bij Duurstede, The Netherlands) and included E11,13-14:Ald (chemical purity 91%; isomeric purity >99%), Z11,13-14:Ald (chemical purity 96%; isomeric purity >99%),

E11,13-14:OH (chemical purity 86%) and E11,13-14:OAc (chemical purity 86%).

2.3 | Observation of female calling behaviour and extraction of pheromone glands

Female *A. comariana* (1–3 day old, $n = 9$) were placed individually in transparent plastic jars and monitored at red light illumination during the whole scotophase, but no extrusion of the pheromone gland could be observed. Without knowledge about the calling period, the initial strategy was to perform extraction 2–3 hr into the scotophase. Abdominal glands of 1–3 day old females were dissected in ultrapure (>99%) heptane (Merck, Darmstadt, Germany) ($\approx 5 \mu\text{l/gland}$) at 22°C for 20 min. Five, seven and eight glands were pooled in separate batches and stored at -18°C until used in electrophysiological and chemical analyses. Additional extractions were performed 1 hr before the end of the photophase as well as 4 hr into the scotophase, but these only contained 1–2 glands.

2.4 | Electrophysiology

Gas chromatography coupled with electroantennographic detection (GC-EAD) was used to identify compounds in *A. comariana* female gland extracts that can be detected by conspecific males. The tip of the antennae of 1–4 day old males were cut off, and the head with antennae mounted to a PRG-2 EAG probe (10× gain) (Syntech, Kirchzarten, Germany) using Blågel conductive gel (Cefar, Malmö, Sweden). For these analyses, aliquots of 0.4 female equivalents of gland extract, and blends of synthetic E11,13-14:Ald, Z11,13-14:Ald, E11,13-14:OH and E11,13-14:OAc (1 ng of each compound), were injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a polar HP-INNOWax column (30 m × 0.25 mm × 0.25 μm film thickness; J&W Scientific, Agilent Technologies, Santa Clara, CA, USA), or a non-polar HP-5 column (30 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific) in splitless mode. Hydrogen was used as carrier gas (flow rate: 1 ml/min), and injector temperature was 250°C. The column effluent was split at a 1:1 ratio between the flame ionization detector (FID) and the EAD. Oven temperature was maintained at 80°C for 1 min after injection, then increased by 5°C/min or 10°C/min to 220°C, with a final hold of 10 min at this temperature. The column effluent passed through a heated transfer line set at 255°C and was mixed with charcoal-filtered and humidified air before reaching the antennal preparation, which was placed 1 cm from the glass tube outlet. Simultaneous FID and EAD signals were recorded using the GC-EAD Pro Version 4.1 software (Syntech). In total, 17 antennal preparations were used in these analyses.

2.5 | Chemical analyses

Compounds in gland extracts that elicited a response in antennae of males were analysed by gas chromatography coupled with mass spectrometry (GC-MS). Extracts were injected into an Agilent

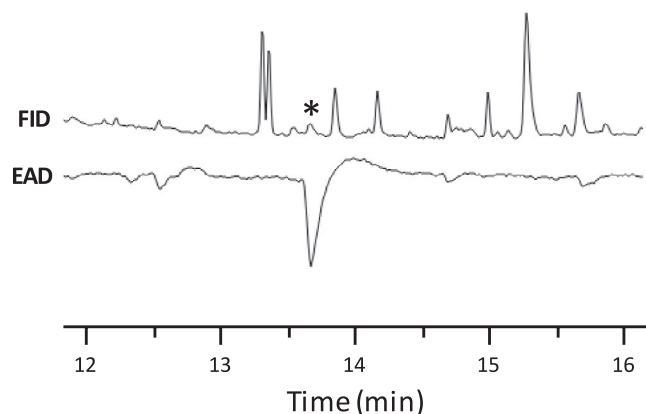


FIGURE 1 Flame ionization detector (FID) and electroantennographic detector (EAD: male *Acleris comariana* antennae) responses to an aliquot of 0.4 female equivalents of pheromone gland extract of *A. comariana* analysed on a HP-INNOWax column. The single EAD-active compound in the extract (*) was identified as (*E*)-11,13-tetradecadienal in subsequent analyses with gas chromatography coupled to mass spectrometry

7890A gas chromatograph equipped with a non-polar HP-5MS column (30 m × 0.25 mm × 0.25 µm film thickness; J&W Scientific) and linked to an Agilent 5975C mass-selective detector. Some analyses were performed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA) equipped with a polar HP-INNOWax column (same dimensions as for the GC-EAD analyses), and linked to a Hewlett-Packard 5975 mass-selective detector. The oven temperature in these analyses was programmed as for the GC-EAD recordings. In all analyses, helium was used as carrier gas (flow rate: 1 ml/min), and injector and transfer line temperatures were 250 and 280°C, respectively. Active compounds were identified through comparison of retention times and mass spectra with those of synthetic reference compounds.

2.6 | Field experiments

To check the attraction of *A. comariana* males to the geometric isomers of 11,13-tetradecadienal, trapping experiments were conducted in the strawberry field near Lockarp during September 2018. Test compounds were dissolved in heptane, and 100 µl of a solution was applied on red rubber septa (11 × 5 mm, #224100-020; Wheaton Science Products, Millville, NJ, USA) to be used as lures in traps. Each test solution contained 1% of the antioxidant 3-tert-Butyl-4-hydroxyanisole (BHA) to stabilize the active ingredient. Transparent plastic delta traps (Csálosom, Budapest, Hungary) with sticky inserts were suspended 1 m above ground from white plastic fence poles (Granngården, Sweden). Five replicates per treatment were used in all experiments. Traps were separated by 10 m within a replicate. The distance between replicates was 10 m in the first experiment, and 50 m in the second and third experiment, and the distance between experiments was at least 100 m. For the last two experiments, three replicates were placed after each other along a row, and the last two replicates in parallel with these. For each

experiment, traps were checked twice per week, captured moths were counted, and inserts replaced if needed. After each check, a trap was moved one step within the row to further reduce positional effects on catches.

In the first experiment, performed 7th to 21st of September, lures included either 100 µg of *E*11,13-14:Ald or only heptane solvent (control). A second experiment was performed 7th to 14th of September, where traps were baited with various ratios of *E*11,13-14:Ald and *Z*11,13-14:Ald (100:0, 75:25, 50:50, 25:75 and 0:100) with a total dose of 100 µg per septum. Finally, a third experiment was performed 14th to 28th of September and tested *E*11,13-14:Ald at 0.1, 1, 10, 100 and 1,000 µg per septum. The traps in this last experiment stayed in the field for 2 weeks, but catches decreased drastically during the second week due to strong winds and low temperatures and many traps were lost, so only catch data from the first week could be included in the statistical analysis. All statistical tests were based on mean weekly catches per trap, which were log ($x + 1$) transformed to approach normal distribution of data. A *t* test was used to compare catches for the first experiment, whereas a one-way ANOVA, followed by multiple comparisons adjusted according to the Bonferroni *post hoc* test, was used to compare catches among treatments for the last two experiments. All tests were performed using SPSS Version 19 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

All moths emerging in the laboratory (≈150 individuals) were identified as *A. comariana*, showing the dominance of this species in strawberry fields in southern Sweden. A subsample of 36 specimens were examined in detail (genital preparation) to confirm species identity using Razowski (2001) and Svensson (2006), and vouchers were saved. At least six colour morphs were observed according to the classification by Turner (1968): grey-brown, grey-black, brown-black, marbled-brown, melanic and plain-brown.

The GC-EAD analyses of gland extractions from 2–3 hr into the scotophase revealed a single compound that consistently elicited a strong response from antennae of males (Figure 1). Subsequent GC-MS analyses of extracts showed that the retention time and mass spectrum of the active compound matched that of synthetic *E*11,13-14:Ald, and the activity of this compound was confirmed in further GC-EAD analyses (Figure 2). The compound was not detected in gland extracts from earlier or later time periods, but these contained few glands. The *Z*-isomer of the aldehyde was also found to be EAD-active (Figure 2). The *E*- and *Z*-isomers could not be resolved on the HP-INNOWax column, and only partially resolved on the HP-5 column using different temperature ramps (0.036 min at 5°C/min, 0.028 min at 10°C/min, and 0.022 min at 15°C/min), making it impossible to distinguish if the antennal response upon stimulation with gland extract was caused by presence of the *E*-isomer alone or both isomers. The presence of the *Z*-isomer, however, could not be confirmed in any of the pooled extracts analysed by GC-MS. Finally, there was no antennal response upon stimulation

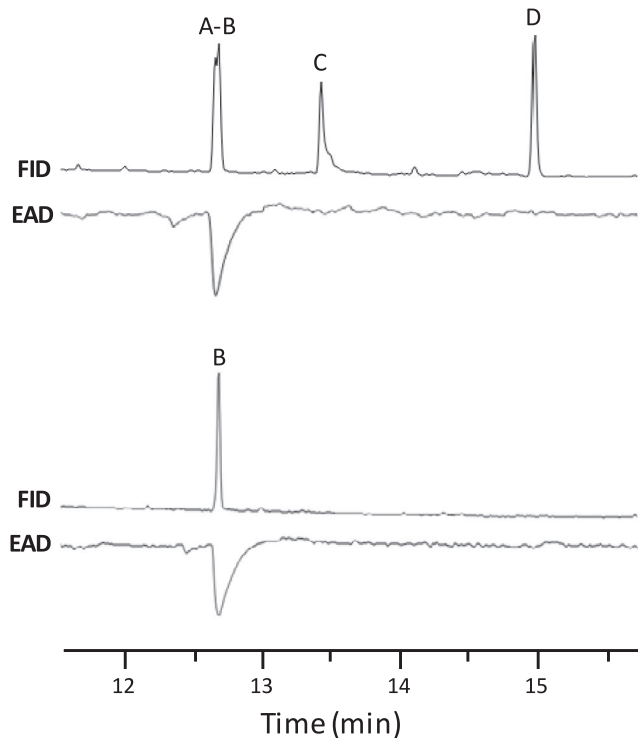


FIGURE 2 Flame ionization detector (FID) and electroantennographic detector (EAD: male *Acleris comariana* antennae) responses to synthetic reference compounds (1 ng of each) analysed on a HP-5 column: (E)-11,13-tetradecadienal (A), (Z)-11,13-tetradecadienal (B), (E)-11,13-tetradecadienol (C) and (E)-11,13-tetradecadienyl acetate (D)

with synthetic E11,13-14:OH or E11,13-14:OAc (Figure 2), and these compounds were not detected in any of the extracts.

In the first field experiment, traps baited with 100 µg of E11,13-14:Ald captured on average 93 males per week, whereas a single male was found in a control trap ($t = 25.10$, $df = 18$, $p < 0.001$, Figure 3). In the second experiment, there was no significant difference in attraction of males to the pure isomers of the aldehyde or blends of these ($F = 1.30$, $df = 4$, $p = 0.31$, Figure 4). Finally, the dose-response experiment showed that catches increased with increasing dose ($F = 165.48$, $df = 4$, $p < 0.001$), and 100 µg and 1,000 µg attracted significantly higher numbers of males compared to 10 µg (Figure 5). The same six colour morphs were found in the traps as observed for the individuals emerging in the climate chamber. No other moth species were captured in significant numbers in any of the three experiments.

4 | DISCUSSION

Infestation by *A. comariana* is an increasing problem in strawberry fields in southern Sweden and Denmark, where this moth is a major pest (Sigsgaard et al., 2014; Svensson, Tönnerberg & Sigsgaard, unpubl. data). Current methods to control populations of this species include treatment with either pyrethroids or *Bacillus thuringiensis*, which is not sufficiently efficient, and pheromone-based methods

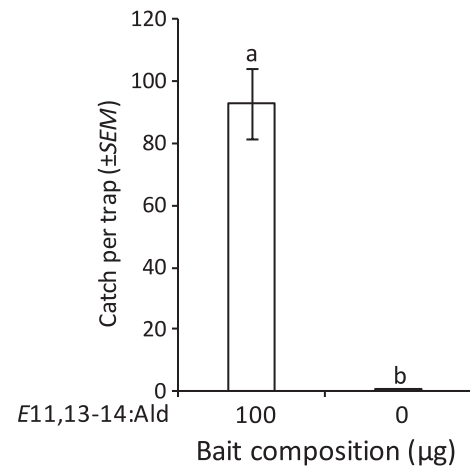


FIGURE 3 Mean catch (\pm SEM) per week of male *Acleris comariana* in traps baited with 100 µg of (E)-11,13-tetradecadienal or heptane solvent (control) in a strawberry field near Lockarp, Sweden, 7–21 September 2018 ($n = 10$). Bars with different letters indicate significantly different catches [t test on $\log(x + 1)$ -transformed data: $p < 0.001$]

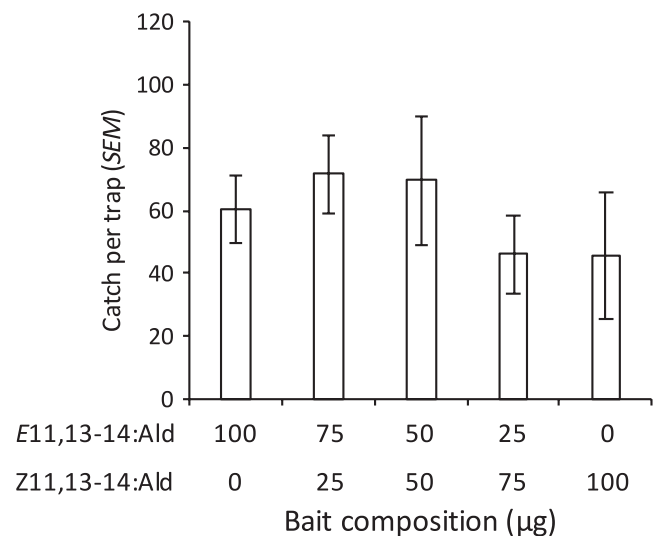


FIGURE 4 Mean catch (\pm SEM) per week of male *Acleris comariana* in traps baited with various ratios of (E)-11,13-tetradecadienal and (Z)-11,13-tetradecadienal (total dose of 100 µg) in a strawberry field near Lockarp, Sweden, 7–14 September 2018 ($n = 5$). No significant differences in catches among treatments were observed [ANOVA on $\log(x + 1)$ -transformed data followed by multiple comparisons according to the Bonferroni post hoc test: $p > 0.05$]

could provide an efficient and sustainable pest management strategy. In this study, we report E11,13-14:Ald as the female-produced sex pheromone of *A. comariana*. No other compound in gland extracts elicited a significant antennal response in males (Figure 1). The Z-isomer of the aldehyde was also found to be antennally active (Figure 2), and attracted similar numbers of males as the E-isomer in the ratio experiment (Figure 4), indicating that Z11,13-14:Ald may also be part of the sex pheromone of *A. comariana*, but it could not

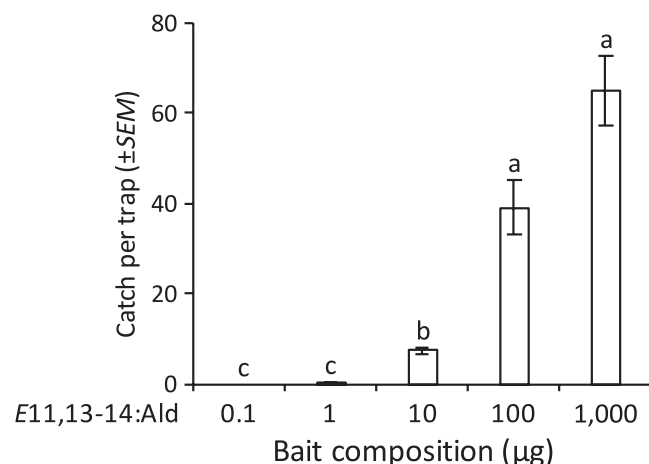


FIGURE 5 Mean catch (\pm SEM) per week of male *Acleris comariana* in traps baited with various doses of (E)-11,13-tetradecadienal in a strawberry field near Lockarp, Sweden, 14–21 September 2018 ($n = 5$). Bars with different letters indicate significantly different catches [ANOVA on $\log(x + 1)$ -transformed data followed by multiple comparisons according to the Bonferroni post hoc test: $p < 0.001$]

be detected in gland extracts in this study. From a practical point of view, isomeric purity of the aldehyde is not critical for efficient monitoring of the species. Traps baited with 100 and 1,000 μ g of E11,13-14:Ald were very efficient in attracting males in a commercial strawberry field (Figure 5). The higher dose trapped on average 66% more males than the lower dose, and the lack of a significant difference in catches between these treatments was probably due to low replication as the experiment terminated after only 1 week.

Our study adds to previous analyses on chemical communication in *Acleris* moths and indicates that E11,13-14:Ald is a key sex pheromone component in this genus. Interestingly, this diene aldehyde has not been reported as part of a sex pheromone in any other lineage of Lepidoptera (www.pherobase.com). Although the compound is a very potent attractant in field experiments of all *Acleris* species investigated, so far it has only been used for monitoring purposes in *A. variaria* in western Canada (Nealis, Silk, Turnquist, & Wu, 2010). This moth is a defoliator of conifers, including western hemlock (*Tsuga heterophylla* (Raf.) Sarg). The study demonstrated the sensitivity of the trapping system in detecting the presence of the pest even at densities below observable damage levels and reported a strong correlation between the number of moth eggs per kilogram fresh foliage and the mean number of captured male moths in pheromone traps. The identified sex pheromone of *A. comariana* has great potential to be used for detection and monitoring of adults as well as predicting damaging levels of larval densities. This would be a considerable improvement over visual observation in terms of reduced workload and earlier detection of the pest.

Few studies have analysed the impact of *A. comariana* in commercial strawberry production. Sigsgaard et al. (2013, 2014) analysed factors determining the abundance of *A. comariana* in Danish strawberry production. Surprisingly, larval densities were

on average four times higher in conventional farms vs. organic farms, and the species was also a more dominant tortricid species in conventional farms (97% of sampled individuals) vs. organic farms (85% of sampled individuals) based on larval counts. In addition, infestation rates were higher in 3 years old fields vs. younger fields, indicating a build-up of populations over time. Direct estimates of the abundance of small and malformed berries in relation to *A. comariana* infestation is difficult to gather because several factors can cause such low-quality berries, for example poor pollination (Andersson, Rundlöf, & Smith, 2012; Muola et al., 2017), transfer of low-quality pollen (Ariza, Soria, Medina, & Martinez-Ferri, 2011) or infestation by other insects, for example other tortricid moths or thrips.

Similar to many other species in the genus *Acleris*, *A. comariana* is very variable in wing colouration, with more than ten described morphs, and the genetic basis for this polymorphism has been revealed in detail by extensive crossing experiments (Fryer, 1928; Turner, 1968). In this study, at least six of these morphs emerged in the laboratory from field-collected larvae sampled from the same strawberry field where the trapping experiments were performed, and the same morphs were found among the captured males. Because no other tortricid species were captured, future monitoring of *A. comariana* could be performed by farmers without previous training in recognizing the different colour morphs of the pest.

Few attempts have been performed to implement pheromone-based methods into pest management in European strawberry production. Cross et al. (2006) evaluated the use of the male-produced aggregation pheromone of the strawberry blossom weevil (*Anthonomus rubi* Herbst) for monitoring and control of this major pest. Traps baited with the pheromone could be used for monitoring, but catches were low, and direct control by means of mass trapping failed. In subsequent studies, combining the aggregation pheromone and the major strawberry floral volatile 1,4-dimethoxybenzene in traps significantly increased catches of *A. rubi* compared to the pheromone alone (Wibe et al., 2014), and lures for *A. rubi* and the European tarnished plant bug (*Lygus rugulipennis* Poppius) were successfully combined in a single trap (Baroffio et al., 2018). Sampson and Kirk (2013) evaluated mass trapping for direct control of the western flower thrips (*Frankliniella occidentalis* (Pergande)) in strawberry fields. Using blue sticky roller traps baited with the aggregation pheromone of the pest reduced the number of adults per flower by 73% and fruit bronzing by 68%, and their cost-benefit analysis revealed this method to be economically viable if implemented into an integrated pest management program of the species.

In summary, we have identified E11,13-14:Ald as the sex pheromone of the strawberry pest *A. comariana*, and showed that this compound is attractive to males. For monitoring of this pest in strawberry fields, a bait containing 100 μ g of E11,13-14:Ald with 1% of BHA as stabilizer provides an efficient trap lure for at least two weeks. The present study is a first step in the development of a pheromone-based monitoring program aiming at tracking the flight

phenology of the spring and summer generations of the species and correlating catch data with estimates of larval density, and possibly also levels of damage, to establish a tool for decision making on direct control measures. Also, future studies should evaluate the potential for direct population control of *A. comariana* via pheromone-based mating disruption.

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AUTHORS' CONTRIBUTIONS

All authors conceived research, GPS and VT conducted experiments and contributed material, GPS analysed data, performed statistical analyses and wrote the manuscript. All authors secured funding, and read and approved the manuscript.

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